

Studying G Protein-Coupled Receptor Internalization Using Live Cell Flow Cytometry

Andrew Huang, Patricia Yeung, Radhika Venkat, Helena Mancebo and Shengwen Zhang*

Multispain, Inc.

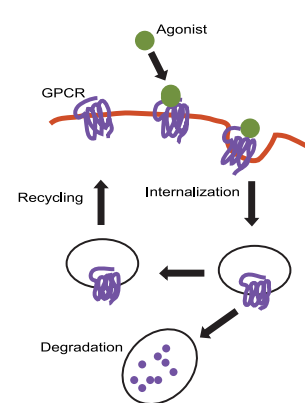
26219 Eden Landing Road, Hayward, CA 94545-3718

*Correspondence: info@multispainc.com

Abstract

G-protein coupled receptors (GPCRs) are among the hottest targets in drug discovery due to their broad involvement in many physiological functions and pathological conditions. We have developed stable mammalian cell lines expressing over 200 GPCRs that are suitable for high throughput cell-based assays including calcium mobilization, cAMP and IP1 assays. However, many GPCRs can couple to different G proteins and therefore activate separate downstream signaling pathways, and more and more compounds have been discovered to selectively activate a subset of these pathways for particular receptors. Screening compounds with one type of readout may generate a significant number of false negatives. In addition, for orphan GPCRs or those without known signal transduction mechanisms, these assays are not effective in identifying receptor activators. On the other hand, many GPCRs undergo ligand-dependent desensitization, a decrease in the responsiveness to ligand, accompanied by receptor aggregation on the cell surface and internalization via clathrin-coated pits to intracellular compartments. Based on this more universal trafficking property of GPCRs, we sought to develop a generalized cell-based drug screening method. Using proprietary recombinant cell technologies, we overexpressed a number of GPCRs with a FLAG tag at their N-terminus and examined their expression on the cell surface. Using live cell flow cytometry, we found that surface expression of the GPCRs decreased upon their agonist treatment in time- and dose-dependent manners, and their pharmacological profiles closely correlated to those obtained by second messenger measurements. Furthermore, the agonist-induced effects can be reversed by receptor-specific antagonists. Our results support that measuring GPCR internalization can be a universal high throughput cell-based approach in identifying drugs that act through the receptors without bias toward any signaling pathway or the knowledge of cellular mechanisms of orphan receptors.

Introduction



Despite the number of new lead compounds being identified for GPCRs through screening by cell-based functional assays, many more are missed because the functional assays used are based on signaling pathways not well suited for the compounds. Other times, it is difficult to establish a screening method due to lack of knowledge of the receptors' signal transduction mechanisms, particularly for orphan receptors. A generalized assay based on the physiological properties of all GPCRs is needed so compounds can be screened regardless of their specificities towards any signal transduction mechanism. One common phenomenon exhibited by GPCRs is receptor aggregation followed by internalization into intracellular compartments, triggered by ligand binding. In this report, we study the properties of human CCK1 receptor internalization in response to ligand treatment using live cell flow cytometry (FACS) and compare with the pharmacological profiles obtained from cAMP functional assays. These results demonstrate that receptor internalization measured by flow cytometry is a valid method for high-throughput drug screening.

Materials and Methods

Cells: CHO-K1 cells stably expressing the human CCK1 receptors.

Compounds: CCK (26-33) was purchased from Phoenix Pharmaceuticals. Lorglumide and Devazepide were purchased from Sigma.

cAMP Assay: Cells treated with compounds were subjected to cAMP assay using cAMP Hi-Range Kit (Cisbio, 62AM6PEC).

Flow Cytometry: Cells were treated with compounds, washed, stained for 30 minutes with an anti-FLAG antibody, and washed again before analysis. Mean Fluorescence Intensity (MFI) data was collected on FACSsort (BD).

Data Analysis: Data was analyzed using GraphPad Prism 4.03.

Human CCK-1 Receptor cAMP Assay

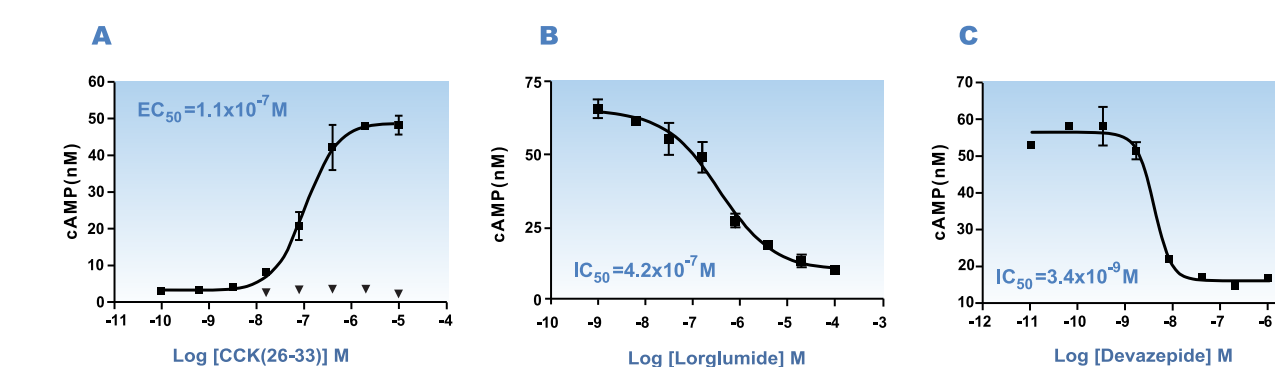


Figure 1. A) Stimulation of cAMP accumulation by agonist CCK (26-33) in cells expressing the human CCK-1 receptor. B) Inhibition of CCK (26-33)-stimulated cAMP accumulation by antagonist lorglumide. C) Inhibition of CCK (26-33)-stimulated cAMP accumulation by antagonist devazepide. The potencies of these compounds are comparable to reported values for the CCK-1 receptor.

Dose-Dependent Receptor Internalization by Agonist

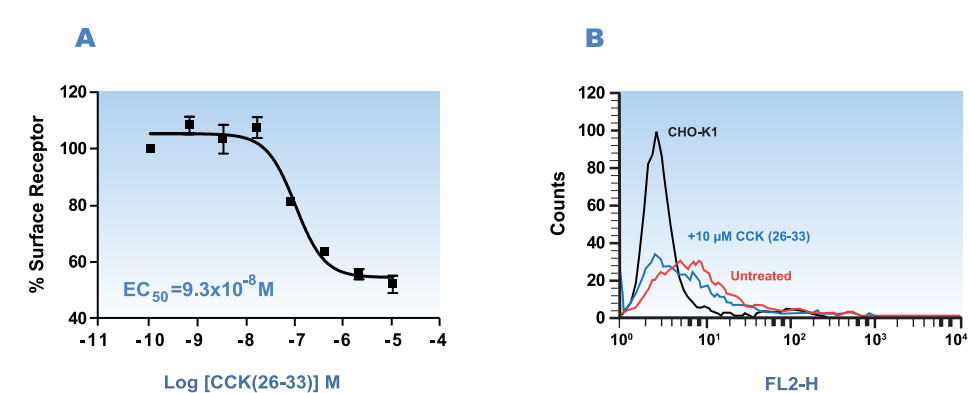


Figure 2. A) CCK1 CHO-K1 cells were treated with agonist CCK (26-33) for 30 minutes, stained with an anti-FLAG antibody and surface expression measured by FACS. Percentage of cell surface receptor was calculated from geometric mean of fluorescence intensities. B) Histogram showing a shift in fluorescence intensity after treatment with the agonist. The potency of CCK (26-33) is comparable to the value acquired by cAMP assay.

Time Course of Receptor Internalization by Agonist

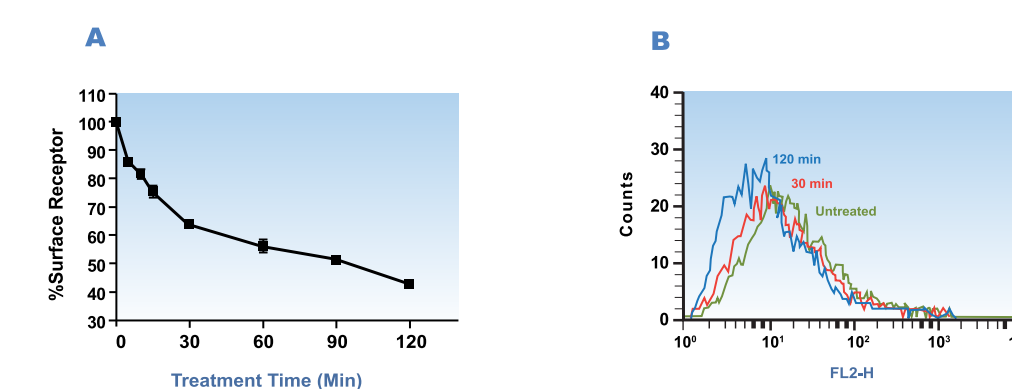


Figure 3. A) CCK1 CHO-K1 cells were treated with 0.27 μM CCK (26-33) (calculated EC₅₀ value based on Figure 2) for various time periods and subjected to FACS analyses. B) Histogram showing shifts in fluorescence intensity after treatment with the agonist. Reduction of surface receptor was evident after 5 minutes of agonist treatment and continued with prolonged treatment.

Dose-Dependent Inhibition of Receptor Internalization by Antagonists

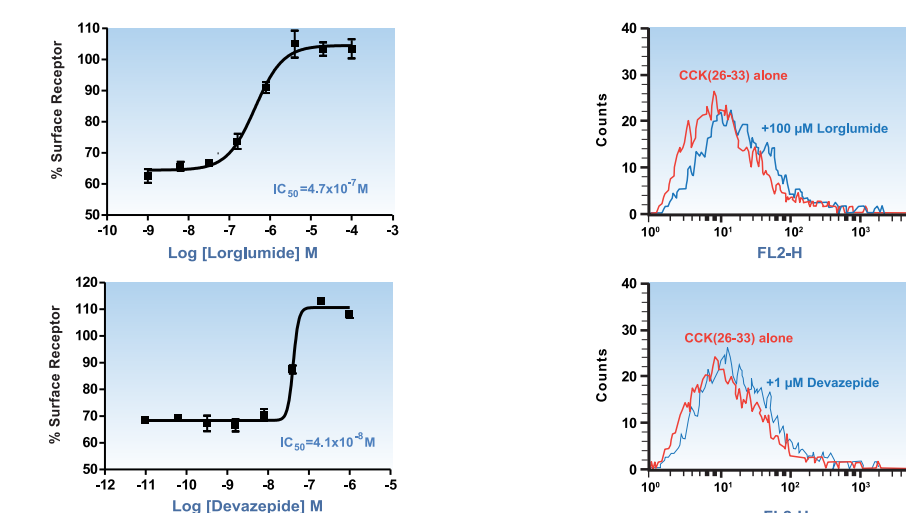


Figure 4. A) CCK1 CHO-K1 cells were treated with antagonists lorglumide or devazepide in the presence of 0.27 μM CCK (26-33) for 30 minutes and subjected to FACS analyses. B) Histograms showing shifts in fluorescence intensity by antagonist treatment. Both CCK-1 receptor antagonists dose-dependently reversed the agonist-induced receptor internalization, with potencies comparable to values acquired by cAMP assays and other reported measurements.

Summary

- The human CCK-1 receptors internalize upon agonist treatment in dose- and time-dependent manners, as measured by cell surface receptor reduction using live cell flow cytometry. The effect of the agonist is reversed by the treatment of receptor-specific antagonists.
- The pharmacological properties of these CCK-1 receptor ligands closely mimic those identified by cAMP assays.
- Measuring receptor internalization by FACS can be a universal high-throughput method to identify drugs that target GPCRs.